Journal of Neuroscience Research 29:401–412 (1991)

Rapid Communication

Cloning of a Human cDNA Expressing a High Voltage-Activating, TEA-Sensitive, Type-A K^+ Channel Which Maps to Chromosome 1 Band p21

B. Rudy, K. Sen, E. Vega-Saenz de Miera, D. Lau, T. Ried, and D.C. Ward
Department of Physiology and Biophysics and Department of Biochemistry, New York University Medical Center, New York, New York (B.R., K.S., E.V.-S.d.M., D.L.), and Department of Human Genetics, Yale University School of Medicine, New Haven, Connecticut (T.R., D.C.W.)

Over ten different mammalian genes related to the *Drosophila* Shaker gene (the Sh gene family) have been identified recently. These genes encode subunits of voltage-dependent K^+ channels. The family consists of four subfamilies: ShI genes are homologues of Shaker; ShII, ShIII, and ShIV are homologues of three other Shaker-like genes in *Drosophila*, Shab, Shaw, and Shal, respectively. We report here the cloning of a human K^+channel ShIII cDNA (HKShIIIC) obtained from a brain stem cDNA library. HKShIIIC transcripts express an atypical voltage-dependent transient (A-type) K^+ current in *Xenopus* oocytes. This current is activated by large membrane depolarizations and is extremely sensitive to the K^+ channel blocker TEA unlike most A-type currents. The gene encoding HKShIIIC maps to chromosome 1p21.

Key words: potassium channels, chromosome 1, Shaker, ShIII subfamily, human genes, brain stem-cerebellum

INTRODUCTION

Ion channels are ubiquitous membrane proteins with important and multiple functions in both excitable and nonexcitable cells (Hille, 1984). Potassium (K^+) channels are particularly diverse and are present in many types of eukaryotic cells (Dubois, 1983; Hille, 1984; Lewis and Cahalan, 1988; Blatz and Magleby, 1987; Rudy, 1988). Because of their diversity and ubiquity K^+ channels participate in a broad spectrum of cellular functions, including excitability, secretion, and osmotic regulation. In neurons specific combinations of various K^+ channels, together with other ion channels, underlie the generation of many different signal waveforms and firing patterns and thus contribute to the complexity of neuronal information coding and integration (Thompson and Aldrich, 1980; Adams and Galvan, 1986; Llinas, 1984, 1988). The elucidation of the molecular basis of K^+ channel diversity will be relevant to many aspects of cell function.

Over ten different mammalian genes related to the *Drosophila* Shaker (Papazian et al., 1987; Baumann et al., 1987; Kamb et al., 1987) gene (the Shaker or Sh gene family) have been identified recently (reviewed in Jan and Jan, 1990; Rudy et al., 1991). These genes encode proteins which are, probably, subunits of voltage-dependent K^+ channels, one of the functional classes (Hille, 1984; Rudy, 1988) of K^+ channels. The family consists of four subfamilies. A member of a subfamily in mammals is much more similar to one of four related *Drosophila* genes (Shaker, Shab, Shaw and Shal; Butler et al., 1989; Wei et al., 1990) than to a mammalian member of a different subfamily, suggesting that precursor genes to each one of the four subfamilies existed prior to the divergence of arthropods and chordates. ShI mammalian genes are homologues of Shaker; ShII of Shab, ShIII of Shaw, and ShIV of Shal (Wei et al., 1990; Jan and Jan, 1990; Rudy et al., 1991). While a single gene is seen in each subfamily in *Drosophila* , multiple **ShI** genes have been described in rodents and humans (Stühmer et al., 1989; Chandy et al., 1990; Tempel et al., 1988; Swanson et al., 1990;

Received March 28, 1991; revised April 24, 1991; accepted April 24, 1991.

Address reprint requests to B. Rudy, Dept. of Physiology, NYU Medical Center, 550 First Avenue, New York, NY 10016.

The sequence reported in this paper has been deposited in the GenBank data base (accession no. M64676).

McKinnon, 1989; Grupe et al., 1990; Ramaswami et al., 1990), indicating that in the chordate line (*Deuterostomia*) the Shaker precursor underwent extensive duplication and subsequent variation. A large number of ShIII gene products have also been identified in rodents, which result from the existence of multiple genes and also from alternative splicing (Yokoyama et al., 1989; McCormack et al., 1990a; Vega et al., 1990, 1991b; Luneau et al., 1991; Sen et al., 1991; Rudy et al., 1991; Schroter et al., 1991). We report here the cloning, functional expression, and chromosomal mapping of HKShIIIC (Sen et al., 1991), a cDNA encoding a human ShIII K^+ channel sequence. HKShIIIC in vitro transcripts express in *Xenopus* oocytes atypical A-type K^+ channels; they activate at high voltages and are extremely sensitive to the K^+ channel blocker tetraethylammonium (TEA), unlike typical A channels which are not blocked by TEA, and usually operate at more negative voltages (Thompson and Aldrich, 1980; Hille, 1984; Rudy, 1988; Solc and Aldrich, 1990).

MATERIALS AND METHODS

Screening of cDNA Libraries

To identify human ShIII cDNAs, two cDNA libraries (1 million recombinants each) in Lambda GT11 derived from human fetal spinal cord and human brain stem (gifts from Dr. Celia Campagnoni, UCLA, and Dr. Carmie Puckett, California Institute of Technology) were screened with two probes (nucleotides 301–501 and 964–1,296) derived from RKShIIIA, a rat ShIII cDNA (McCormack et al., 1990a). Hybridization to nitrocellulose replicas of cultures (Maniatis et al., 1982) was carried out in 30% formamide, 5X SSPE, at 40°C; the filters were washed in 0.2 × SSC with 0.1% SDS at 45°C. DNA was extracted from purified positive phage plaques after amplification. The DNA was cut with EcoR1 and the inserts gel purified and subcloned into pBluescript (Strategene). We obtained several human ShIII cDNAs from these screens. Three plasmid cDNA clones, 2C, 6A, and 32-2, derived from phage plaques purified from the brain stem library contained HKShIIIC inserts. The sequence analysis and the functional expression studies shown here correspond to the cDNA clone 2C. 2C and 6A were used for the in situ hybridization studies. 6A and 32-2 contain only part of the apparent coding sequence of HKShIIIC but extend about 1,000 bases on the 3′ end.

DNA Sequencing

Sequences were obtained by the dideoxynucleotide chain termination method (Sanger et al., 1977) using Sequenase™ (U.S. Biochemicals) and plasmid DNA as template. Both strands were sequenced using both the nested deletions method (Henikoff, 1984; using a protocol from Promega Protocols and Applications Guide; Promega, Madison, WI) and synthetic oligonucleotide primers to specific internal sequences.

RNA Expression

The recombinant Bluescript plasmid (Stratagene) containing the HKShIIIC insert was linearized by digestion with XbaI and full-length capped RNA transcripts (cRNA) synthesized with T3 polymerase as previously described (Iverson and Rudy, 1990). The cRNA was stored in distilled water at 200 ng/μl at −70°C. Stage V and VI *Xenopus laevis* oocytes were prepared as in Iverson and Rudy (1990) except that usually the oocytes were not defolliculated. The oocytes were injected with 2–5 ng of cRNA/oocyte and incubated for 2 to 3 days at 20°C in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5) supplemented with 2.5 mM sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin. Prior to electrophysiological recording the oocytes were defolliculated manually after a brief (5 min) treatment with collagenase (Type I Sigma at 1 mg/ml). All electrophysiological recordings were carried out at 21–22°C with a standard two microelectrode voltage-clamp (Iverson and Rudy, 1990) under continuous perfusion with ND96. The data were low-pass filtered at 3 kHz with an 8-pole Bessel filter and digitized and analyzed using the pCLAMP system (Axon Instruments).

In Situ Hybridization

The chromosomal localization of HKShIIIC was determined by fluorescence in situ hybridization. Probe labeling and in situ hybridization followed protocols described previously (Lichter et al., 1990; Ried et al., 1990). Briefly, DNA from two plasmids (2C and 6A) containing two distinct HKShIIIC inserts was labeled with biotin-11-dUTP (Sigma) in a nick translation reaction. An Alu-PCR probe, to produce an R-like banding pattern, was generated as described in Baldini and Ward (1991) and labeled with digoxigenin-11-dUTP (Boehringer Mannheim) by nick translation. 30 ng of one cDNA probe and 30 ng of Alu-PCR products were combined and hybridized simultaneously as described (Baldini and Ward, 1991) to metaphase spreads prepared following the methotrexate synchronization procedure (Yunis, 1976). Following overnight incubation, posthybridization washes, and a blocking step, the biotinylated cDNA probe was detected with avidin-DCS-fluorescein (Vector), the digoxigenin Alu-PCR products with anti-digoxigenin conjugated to TRITC (Boehringer Mannheim). The R-like banding pattern and the signal from the cDNA probe were imaged separately using a Zeiss axioskop epifluorescence microscope equipped with a cooled CCD camera (Photometrics). The camera was

controlled and images were merged using an Apple McIntosh IIX computer with a software program developed by Tim Rand in D.C. Ward's laboratory. Pictures were taken directly from the screen with a Nikon F3 camera using Kodak 100HC color slide film.

RESULTS

Primary Structure of HKShIIIC

The nucleotide and deduced amino acid sequence of HKShIIIC is shown in Figure 1A. The predicted product of the HKShIIIC cDNA is a protein of 582 amino acids with a calculated relative mass of 64,526. A hydropathy analysis (Kyte and Doolittle, 1982) of the deduced protein is presented in Figure 1B. Like other Sh proteins, the predicted product of HKShIIIC contains a core region with six hydrophobic sequences (H1, H2, H3, H4, H5, and H6) of which five (H1, H2, H3, H4, and H6) are long enough to span the membrane as an α helix and are considered probable membrane spanning domains (Catterall, 1988; Guy, 1990; Jan and Jan, 1990; Rudy et al., 1991). Between the third and fourth hydrophobic segments there is a sequence, thought to be membrane spanning, consisting of an arginine (sometimes lysine) at every third position and neutral, mainly hydrophobic, amino acids in the other positions. This motif, believed to be responsible for voltage sensing, is known as S4 and is also present in voltage-dependent Na^+ and Ca^{++} channels (Noda et al., 1986; Numa, 1987; Tanabe et al., 1987; Catterall, 1988). The amino acid sequence of HKShIIIC is compared to that of two other ShIII sequences in Figure 2. HKShIIIC shows 70% amino acid identity with RKShIIIA and 73% with RKShIIIB. HKShIIIC shows 49% amino acid identity to the *Drosophila* Shaw gene and ~40% to the products of ShI, ShII, and ShIV genes in a core region used to compare Sh proteins that includes all the membrane portion of the polypeptides (marked with arrows in Fig. 2). These values are similar to those seen in other ShIII proteins (Yokoyama et al., 1989; McCormack et al., 1990a; Vega et al., 1991b).

HKShIIIC shows several structural features common to all known ShIII proteins: (1) a long stretch of amino acid sequence in the amino end region which is nearly identical to an equivalent stretch in the *Drosophila* Shaw gene (McCormack et al., 1990a; Yokoyama et al., 1989; Vega et al., 1991; Schroter et al., 1991; shown in brackets in Fig. 2); (2) two putative N-glycosylation sites in the linker between the H1 and H2 domains, which is thought to face the extracellular surface of the membrane, (indicated with triangles in Fig. 2); (3) six positive charges in the S4 domain (boxed in Fig. 2); (4) a phenylalanine in the fourth position of the "leucine-heptad-repeat" (indicated with filled circles in Fig. 2) of Sh proteins (McCormack et al., 1989; McCormack et al., 1991); (5) a small insert in between the H4 and H5 domains (approximately between residues 409 and 417 in HKShIIIC) absent in other Sh proteins. The amino acid sequence of this insert is different in HKShIIIC and in the other two sequences shown in Figure 2; (6) a tyrosine in the H5 domain in a position where this residue produces high sensitivity to TEA (MacKinnon and Yellen, 1990). The residue two positions C-terminal to the tyrosine in HKShIIIC has a *lysine* while RKShIIIA and RKShIIIB have a *glutamine*; and (7) a cluster of positive residues after the H6 domain (indicated with a star in Fig. 2).

A comparison of RKShIIIA with other Sh proteins reveals a 44 aa long insert (McCormack et al., 1990a) in the amino end region of the protein (residues 56 to 99 in RKShIIIA.1 in Fig. 2), which is expected to be intracellular in the assumed topology of Sh channels. The sequence contains several stretches of consecutive prolines and two serines that may undergo O-glycosylation (McCormack et al., 1990a). In the equivalent position of HKShIIIC there is a shorter sequence rich in glycines.

A significant difference between HKShIIIC and RKShIIIA and B is a 28 amino acid insert at the amino end of the protein. The existence of this insert depends on the choice of start ATG (Fig. 1) and could be of physiological significance (see below).

The predicted protein product of HKShIIIC is very similar to a rat ShIII protein (Raw3) published while this manuscript was under preparation (Schroter et al., 1991). Both proteins show 96% amino acid identity up to Ala-538 and then diverge completely. Although this divergence could reflect species differences an alternative possibility should be considered. The divergence starts immediately after an **AG** in HKShIIIC (marked with an arrow in Fig. 1), the dinucleotide characteristic of 5′ splice (or donor) junctions (Mount, 1982). Alternative splicing has been suggested as a mechanism to generate several products with different carboxyl ends in two other rat ShIII genes (Luneau et al., 1991; Sen et al., 1991; Vega et al., 1991b). It is thus possible that Raw3 and HKShIIIC are alternatively spliced variants of one ShIII gene.

Functional Properties of HKShIIIC Channels

Xenopus oocytes injected with HKShIIIC in vitro transcripts express transient outward currents absent in uninjected oocytes (Fig. 3). The reversal potential of these currents, determined from tail current analysis (Iverson et al., 1988), depends on external K^+ concentration as expected for a K^+ selective channel. A reversal potential of −85 mV is obtained in 2 mM external K^+. A plot of reversal potentials as a function of external K^+ concentration (between 10 and 100 mM; data not shown) gives a slope of 52 mV for a ten-fold change in

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    -156
    GGGAGG  TGGTTGGGGC  AAGCCCAAGC  CGCAGAGGGG  GCCGCCACCG  CCTCCTGCCT

-100
CCTCTTCGTC  TCCTCCCCCT  CCCCCGTCTG  ACGCTGCCTC  CTCGGGAAGG  GTGTTTGGAG

-40
GGCAGCGGCC  GCCCCAAGCC  GGAGACCCGC  AGCGCTTCTT  ATG ATC AGC TCG GTG TGT    18
                                                Met Ile Ser Ser Val Cys     6

GTC TCC TCC TAC CGC GGG CGC AAG TCG GGG AAC AAG CCT CCG TCC AAA ACA TGT    72
Val Ser Ser Tyr Arg Gly Arg Lys Ser Gly Asn Lys Pro Pro Ser Lys Thr Cys    24

CTG AAG GAG GAG ATG GCC AAG GGC GAG GCG TCG GAG AAG ATC ATC ATC AAC GTG   126
Leu Lys Glu Glu Met Ala Lys Gly Glu Ala Ser Glu Lys Ile Ile Ile Asn Val    42

GGC GGC ACG CGA CAT GAG ACC TAC CGC AGC ACC CTG CGC ACC CTA CCG GGA ACC   180
Gly Gly Thr Arg His Glu Thr Tyr Arg Ser Thr Leu Arg Thr Leu Pro Gly Thr    60

CGC CTC GCC TGG CTG GCC GAC CCC GAC GGC GGG GGC CGG CCC GAG ACC GAT GGC   234
Arg Leu Ala Trp Leu Ala Asp Pro Asp Gly Gly Gly Arg Pro Glu Thr Asp Gly    78

GGC GGT GTG GGT AGC AGC GGC ACG AGC GGC GGC GGG GGC TGC GAG TTC TTC TTC   288
Gly Gly Val Gly Ser Ser Gly Thr Ser Gly Gly Gly Gly Cys Glu Phe Phe Phe    96

GAC AGG CAC CCG GGC GTC TTC GCC TAC GTG CTC AAC TAC TAC CGC ACC GGC AAG   342
Asp Arg His Pro Gly Val Phe Ala Tyr Val Leu Asn Tyr Tyr Arg Thr Gly Lys   114

CTG CAC TGC CCC GCG GAC GTG TGC GGG CCG CTC TTC GAA GAG GAG CTC ACC TTC   396
Leu His Cys Pro Ala Asp Val Cys Gly Pro Leu Phe Glu Glu Glu Leu Thr Phe   132

TGG GGC ATC GAC GAG ACC GAC GTG GAA CCC TGC TGC TGG ATG ACC TAC CGG CAG   450
Trp Gly Ile Asp Glu Thr Asp Val Glu Pro Cys Cys Trp Met Thr Tyr Arg Gln   150

CAC CGC GAC GCC GAG GAG GCG CTC GAC ATC TTC GAG AGC CCG GAC GGA GGC GGC   504
His Arg Asp Ala Glu Glu Ala Leu Asp Ile Phe Glu Ser Pro Asp Gly Gly Gly   168

AGC GGC GCG GGG CCC AGC GAC GAG GCC GGC GAC GAT GAG CGG GAG CTG GCC CTG   558
Ser Gly Ala Gly Pro Ser Asp Glu Ala Gly Asp Asp Glu Arg Glu Leu Ala Leu   186

CAG CGA CTG GGC CCC CAC GAG GGA GGC GCG GGC CAT GGC GCC GGG TCT GGG GGC   612
Gln Arg Leu Gly Pro His Glu Gly Gly Ala Gly His Gly Ala Gly Ser Gly Gly   204

TGC CGC GGC TGG CAG CCC CGC ATG TGG GCG CTC TTC GAG GAT CCC TAC TCC TCC   666
Cys Arg Gly Trp Gln Pro Arg Met Trp Ala Leu Phe Glu Asp Pro Tyr Ser Ser   222

                                        H1
CGG GCC GCT AGG GTA GTG GCC TTT GCC TCT CTC TTC TTC ATC CTG GTC TCC ATC   720
Arg Ala Ala Arg Val Val Ala Phe Ala Ser Leu Phe Phe Ile Leu Val Ser Ile   240

                                                    ■
ACC ACT TTC TGC CTG GAG ACC CAT GAG GCC TTT AAT ATC GAC CGC AAC GTG ACA   774
Thr Thr Phe Cys Leu Glu Thr His Glu Ala Phe Asn Ile Asp Arg Asn Val Thr   258

                ■
GAG ATC CTC CGC GTA GGG AAC ATC ACC AGC GTC CAC TTC CGG CGG GAG GTA GAG   828
Glu Ile Leu Arg Val Gly Asn Ile Thr Ser Val His Phe Arg Arg Glu Val Glu   276
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                                                                H2
ACA GAG CCC ATC CTG ACC TAC ATC GAG GGC GTA TGT GTG CTG TGG TTC ACA CTG   882
Thr Glu Pro Ile Leu Thr Tyr Ile Glu Gly Val Cys Val Leu Trp Phe Thr Leu   294

GAG TTC CTG GTG CGC ATC GTG TGC TGC CCC GAC ACG CTG GAC TTC GTC AAG AAC   936
Glu Phe Leu Val Arg Ile Val Cys Cys Pro Asp Thr Leu Asp Phe Val Lys Asn   312

                                                        H3
CTG CTC AAC ATC ATC GAC TTT GTG GCC ATC CTG CCC TTC TAC CTG GAG GTG GGA   990
Leu Leu Asn Ile Ile Asp Phe Val Ala Ile Leu Pro Phe Tyr Leu Glu Val Gly   330

CTG AGC GGC CTG TCA TCC AAG GCG GCC CGC GAC GTG CTG GGC TTC CTG CGC GTG  1044
Leu Ser Gly Leu Ser Ser Lys Ala Ala Arg Asp Val Leu Gly Phe Leu Arg Val   348

                    S4
GTG CGC ATC GTG CGC ATC CTG CGT ATC TTC AAG CTC ACA CGC CAC TTC GTG GGG  1098
Val Arg Ile Val Arg Ile Leu Arg Ile Phe Lys Leu Thr Arg His Phe Val Gly   366

CTA CGC GTG CTG GGC CAC ACC CTG AGG GCC AGC ACC AAT GAG TTC CTG CTG CTT  1152
Leu Arg Val Leu Gly His Thr Leu Arg Ala Ser Thr Asn Glu Phe Leu Leu Leu   384

                        H4
ATC ATC TTC CTG GCC CTG GGT GTG CTC ATC TTT GCC ACC ATG ATC TAC TAC GCT  1206
Ile Ile Phe Leu Ala Leu Gly Val Leu Ile Phe Ala Thr Met Ile Tyr Tyr Ala   402

GAG CGC ATT GGG GCC AGG CCC TCC GAC CCT CGG GGT AAT GAC CAC ACC GAC TTC  1260
Glu Arg Ile Gly Ala Arg Pro Ser Asp Pro Arg Gly Asn Asp His Thr Asp Phe   420

                                                            H5
AAG AAC ATC CCC ATT GGC TTC TGG TGG GCT GTG GTC ACC ATG ACG ACA CTG GGC  1314
Lys Asn Ile Pro Ile Gly Phe Trp Trp Ala Val Val Thr Met Thr Thr Leu Gly   438

TAC GGA GAC ATG TAC CCC AAG ACG TGG TCA GGC ATG CTG GTA GGG GCA CTG TGT  1368
Tyr Gly Asp Met Tyr Pro Lys Thr Trp Ser Gly Met Leu Val Gly Ala Leu Cys   456

                H6
GCA CTG GCT GGC GTG CTC ACC ATC GCC ATG CCG GTG CCT GTC ATC GTC AAC AAC  1422
Ala Leu Ala Gly Val Leu Thr Ile Ala Met Pro Val Pro Val Ile Val Asn Asn   474

TTC GGC ATG TAC TAC TCC CTG GCC ATG GCC AAG CAG AAG CTG CCC AAG AAA CGG  1476
Phe Gly Met Tyr Tyr Ser Leu Ala Met Ala Lys Gln Lys Leu Pro Lys Lys Arg   492

AAG AAG CAC GTG CCA CGG CCG GCG CAG CTG GAG TCA CCC ATG TAC TGC AAG TCT  1530
Lys Lys His Val Pro Arg Pro Ala Gln Leu Glu Ser Pro Met Tyr Cys Lys Ser   510

GAG GAG ACT TCC CCC CGG GAC AGC ACC TGC AGT GAT ACC AGC CCC CCT GCC CGG  1584
Glu Glu Thr Ser Pro Arg Asp Ser Thr Cys Ser Asp Thr Ser Pro Pro Ala Arg   528

                                          ↓
GAA GAG GGT ATG ATC GAG AGG AAA CGG GCA GGT GAG ATT AGG GGT TGG GAA GGA  1638
Glu Glu Gly Met Ile Glu Arg Lys Arg Ala Gly Glu Ile Arg Gly Trp Glu Gly   546

AAA TCC CTT TTC CCC CAG TGG CCT AGG GAG TTT CCA AAT GGA CCT CAG ACC TTG  1692
Lys Ser Leu Phe Pro Gln Trp Pro Arg Glu Phe Pro Asn Gly Pro Gln Thr Leu   564

GGA TTT GGC ATG TGT TTT GTG TGG GGC TTC CCT AAG CAT AAA GAT GTG CCT TTA  1746
Gly Phe Gly Met Cys Phe Val Trp Gly Phe Pro Lys His Lys Asp Val Pro Leu   582

TGA G  GGCAAAGTGT  TAAAAAAAAA  AAAAAAAAAA  AA                            1782
*
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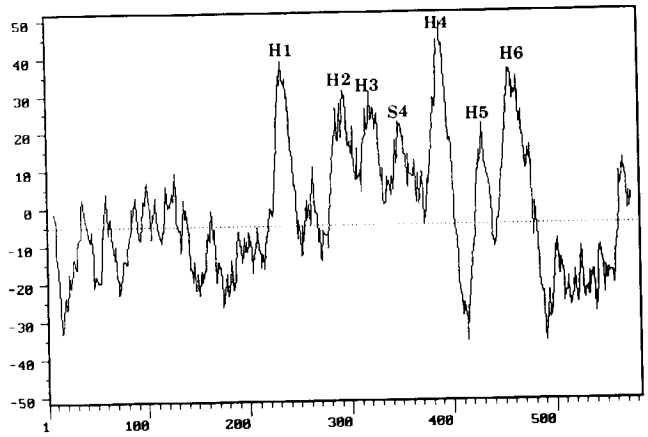
A



B

Fig. 1. **A:** Nucleotide- and deduced amino-acid sequence of HKShIIIC (clone p2C). The name given to this clone is based on the nomenclature used by McCormack et al. (1990a), which attempts to indicate its relationship to other K^+ channel sequences. The H indicates the clone is from human, K that it is a K^+ channel, Sh that it belongs to the Sh family of genes, III that it is a member of the third gene subfamily, and C the third member of this subfamily. One may use the term KShIIIC as a shorthand when it is not necessary to specify species. The numbers indicate the nucleotide and amino-acid positions with the chosen initiation codon as 1. This is the first ATG resulting in a long open reading frame and predicts a protein that is highly similar to other Sh K^+ channel sequences. A second, in frame, ATG is found 84 nucleotides downstream. Neither of these two ATGs is surrounded by a particularly good consensus sequence for translation initiation (Kozak, 1989). Although the second ATG does have a purine in position −3 and a G in position +4, the first ATG is used here until this is clarified experimentally. The first stop codon in frame is shown by an asterisk. The two potential start codons are underlined. The hydrophobic domains (H1–H6) and the S4 motif are overlined. Asparagines surrounded by consensus sequences for N-glycosylation (Marshall, 1974) are indicated with a filled square. These sites are extracellular in the expected topology of the protein, which assumes that the amino-terminus is intracellular (because of a lack of signal peptide sequence) and that H1 to H4, S4, and H6 are membrane spanning domains (Jan and Jan, 1990; Catterall, 1988; Guy 1990; Rudy et al., 1991). An arrow indicates the point of divergence between HKShIIIC and Raw3 (Schroter et al., 1991). **B:** Hydropathy analysis of the predicted translation product of HKShIIIC by the method of Kyte and Doolittle (1982) with an interval of 15 amino-acids. The relative hydrophobicity index is shown on the ordinate and the amino-acid positions on the abscissa. The H1–H6 and S4 domains are indicated.

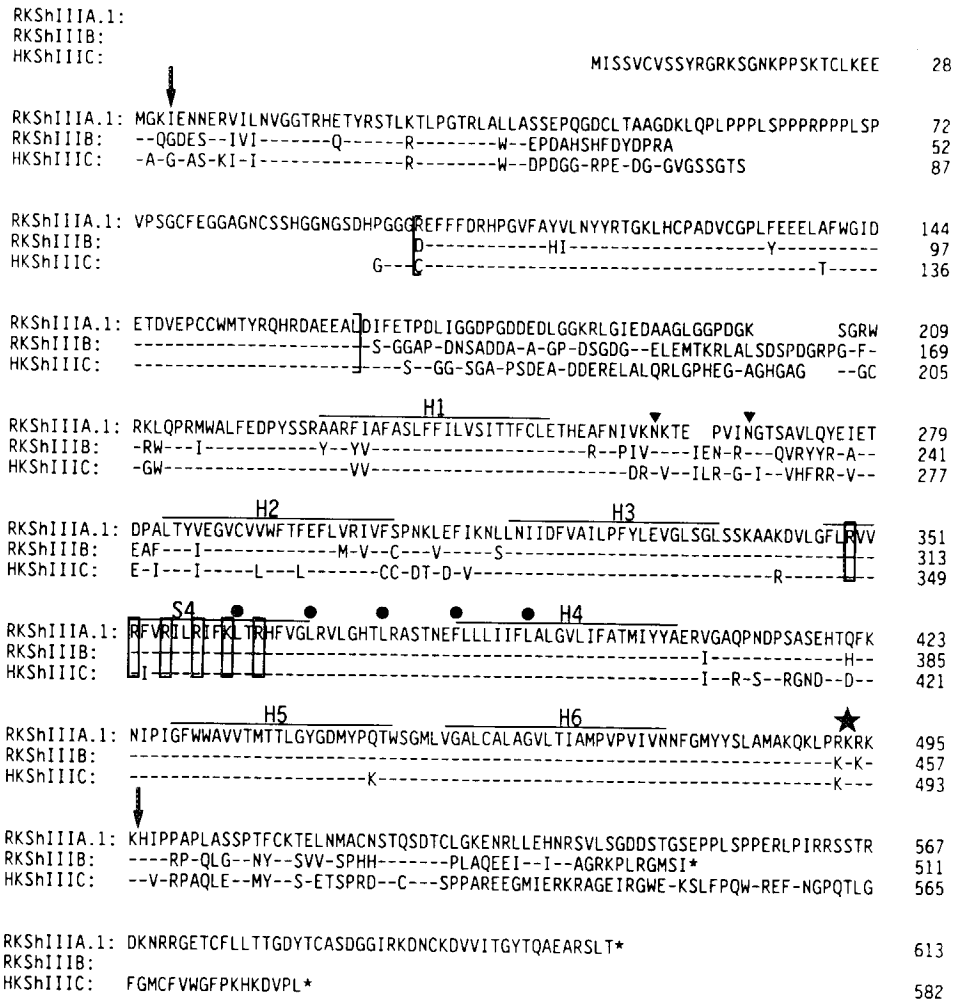


Fig. 2. Comparison of predicted amino acid sequences of three ShIII genes. RKShIIIA.1 is one of three alternatively spliced products of RKShIIIA (McCormack et al., 1990a; Vega et al., 1991b). RKShIIIB was cloned from PC12 cell libraries (Vega et al., 1991a) and is probably a rat homologue of NGK2 (Yokoyama et al., 1989). The sequences were aligned to maximize long stretches of homology. Identical amino acids are shown with a dash. Gaps required for optimized alignment of the sequences are shown as blanks. The beginning and the end of the central core region used for comparisons of identity with other K^+ channel proteins (e.g., Frech et al., 1989; McCormack et al., 1990a; Ramaswami et al., 1990) are marked by arrows. The H1 to H6 and S4 sequences are overlined. The stretch in the amino end region of the protein that is nearly identical to an equivalent stretch in the *Drosophila* Shaw gene is shown in brackets. The positive charge residues in S4 have been boxed. The leucines in the Sh leucine heptad repeat adjacent to the S4 domain are indicated with a filled circle. The fourth residue in the repeat is phenylalanine in ShIII proteins, but leucine in all other Sh proteins (Rudy et al., 1991).

external K^+ concentration. This value is somewhat smaller than that predicted (56 mV) by the Nernst equation for a purely K^+-selective channel and indicates some permeability to other ions (Hille, 1984).

Several properties of HKShIIIC currents are presented in Figure 3. The currents start activating at about −10 mV (Fig. 3A,B), they rise relatively fast, reach a maximum, and then quickly inactivate. The time for the currents to reach a maximum after the initiation of a depolarization (time to peak) decreases with increasing depolarization, approaching a minimum value at high depolarizations (Fig. 3C). At +40 mV the average time to peak for HKShIIIC currents is 10.2 ± 0.7 (n = 5) msec, about 2–3 times faster than the rise time of RKShIIIA or RKShIIIB currents at the same potential (McCormack et al., 1990a; Vega et al., 1991). The rate at which the current declines or inactivates during the depolarizing pulse is also voltage dependent, increasing with increasing depolarization (Fig. 3D). This is a property of some but not all fast-inactivating K^+ currents (Connor and Stevens, 1971a; Rudy et al., 1988; Rudy, 1988; Solc and Aldrich, 1990).

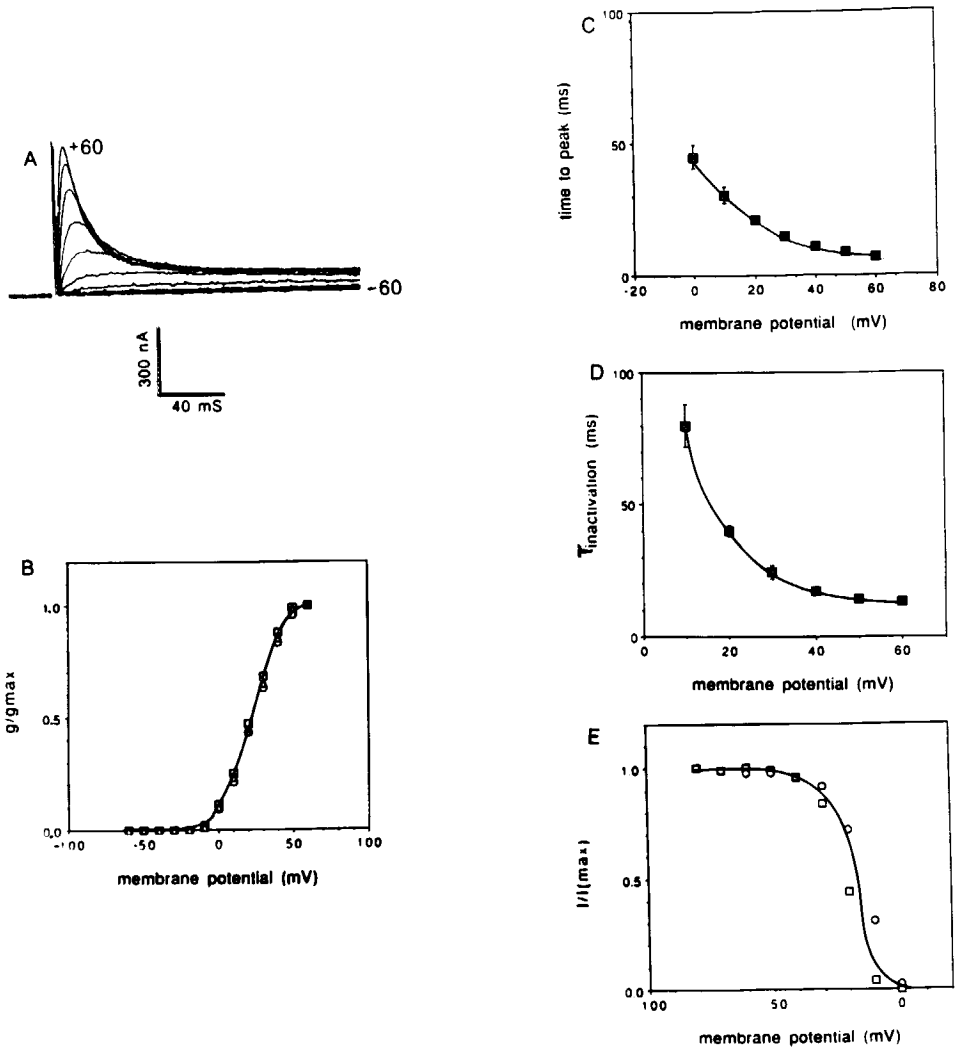


Fig. 3. Electrophysiological properties of ion currents expressed by RNA transcripts of HKShIIIC. **A:** Currents recorded under voltage clamp. Depolarizing pulses, from −60 to +60 mV in 10 mV increments, were delivered at a rate of every 10 sec from a holding potential of −100 mV. The oocytes were bathed in ND96 solution and the electrodes filled with 3 M KCl. **B:** Plot of normalized conductance (conductance at the indicated potential divided by the maximum conductance) obtained from three oocytes, and two different preparations of RNA. The conductance was calculated by dividing the peak current at a given potential by the driving force using a reversal potential of −85 mV. **C:** Voltage dependence of the rise time of HKShIIIC currents. The time required for the currents to reach their maximum value from the initiation of the depolarization (time to peak) as a function of membrane potential. Shown is the average from five experiments. Error bars are standard deviations. **D:** Voltage dependence of the time constants of inactivation of HKShIIIC currents. The decline of the currents at the indicated potential, to a steady state value, was fitted to a single exponential. These fits followed the decay of the currents quite closely. Shown is the average of five experiments. Error bars are standard deviations. **E:** Voltage dependence of steady state inactivation of HKShIIIC currents. Shown is the peak current during a depolarizing pulse to +50 mV preceded by a 1-sec prepulse to the voltage indicated in the abscissa (I) over the peak current during a depolarizing pulse to +50 mV preceded by a 1-sec prepulse to −100 mV (I_{max}). The data is from two different oocytes.

A peak conductance vs. voltage curve obtained from three experiments is presented in Figure 3B. The conductance begins to rise at about −10 mV and increases steeply to a maximum value. Half maximal conductance is obtained at 19.1 ± 2.3 mV (SD, n = 5) and the slope of the curve is estimated to be −11.3 ± 1.16 mV for an e-fold change in conductance. The midpoint of the conductance-voltage curve is about 5 mV more

positive than that of RKShIIIA and B currents, and its slope less steep. However, in comparing these properties with those of other Sh channels it should be taken into account that these are inactivating currents and that the peak current does not necessarily reflect the maximum probability of channel opening at a given membrane potential. As a result of inactivation the peak-conductance vs. voltage curve is likely to be shifted to the right and its slope decreased to the curve reflecting open channel probability vs. voltage for the same channels. (Zagotta and Aldrich, 1990). Single channel studies will be required however to confirm this.

Figure 3E shows data to determine the voltage-dependence of steady state inactivation taken from two different oocytes. The data were fitted to a Boltzmann distribution with e-fold increase in inactivation for 7.2 mV. HKShIIIC channels are half inactivated at −15 mV. The fast inactivation of HKShIIIC currents defines them as A-type currents (Rudy, 1988; Solc and Aldrich, 1990); however, HKShIIIC currents activate and inactivate at voltages much higher than the currents discovered in molluscan neurons and later found to be present in other cells including in mammalian neurons, for which the term "A" currents was first coined. The original A currents activate and inactivate at very negative potentials, close or sometimes more negative than the membrane resting potential, and thus operate in the subthreshold region for action potential generation and play important roles in regulating firing frequencies (Connor and Stevens, 1971a,b; Adams and Galvan, 1986; Dekin and Getting, 1984; Llinas, 1984; Hille, 1984; Rudy, 1988). However, the term has now been extended to all fast inactivating voltage-gated K^+ currents (see discussion on "A" currents in Rudy, 1988; and introduction in Solc and Aldrich, 1990).

Since the original observations of Thompson (1977) in molluscan neurons, 4-Amino pyridine (4AP) is commonly used to block A currents, although A currents are not the only or the most 4AP-sensitive K^+ currents (Rudy, 1988). Like other A currents, HKShIIIC currents are blocked by 4AP at mM concentrations. Half block of HKShIIIC currents is seen at 0.6 ± .12 mM (SD n = 4; data not shown). However, unlike most other A currents, but like other ShIII currents, HKShIIIC currents are also blocked by low concentrations of externally applied TEA (Fig. 4). Half block of HKShIIIC currents is obtained at 88 ± 12 μM TEA (SD, n = 6).

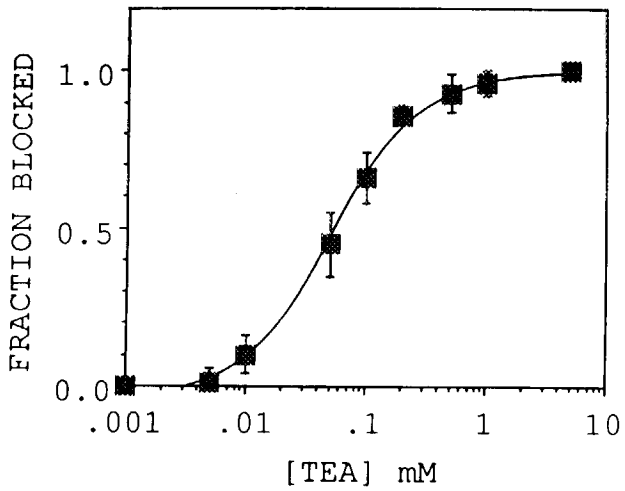


Fig. 4. Dose response of HKShIIIC current block by TEA. The fraction of the current (at +50 mV) blocked by TEA (1 − the ratio of the peak current after perfusion with TEA over the peak current before the change of solution) is plotted as a function of the concentration of applied TEA. Shown are the averages from six different experiments in different oocytes. Error bars are standard deviations. Tetraethylammonium chloride (Eastman) was diluted fresh in ND96 from a 1 M, filtered, stock solution stored at −20°C. TEA was continuously perfused and measurements taken after the current magnitude did not change further.

Chromosomal Mapping

The chromosomal mapping of HKShIIIC cDNA clones was determined by fluorescence in situ hybridization. The cDNA clones were labeled with biotin and hybridized to human metaphase chromosomes. The signal was detected with avidin-fluorescein. To assign the position of the cDNA clones with respect to cytogenetically defined bands, we used the recently described in situ hybridization banding with Alu-PCR products (Baldini and Ward, 1991). This hybridization banding results in an R-banding pattern of human chromosomes, corresponding to conventional R-banding. Cohybridization of biotinylated probe-DNA and digoxigenin labeled Alu-PCR products, which are detected with a second fluorochrome, facilitates the band assignment considerably. The images were taken subsequently using the appropriate filter combination and were merged and pseudocolored electronically resulting in a two-color picture, which allows the precise localization of the cDNA clones to band p21 of chromosome 1 (Fig. 5). Two overlapping cDNAs (2C and 6A) gave the same results. Due to the possible occurrence of false (background) signals or other potential technical artifacts, it is necessary to show that the position of the hybridization signal is highly reproducible. In the case of multigene families false hybridization signals may also result from cross-hybridization to related genes. A total of 12 metaphase spreads were investigated: eight revealed two hybridization signals on both homologues of chromosome 1 at p21, three showed two hybridization signals on only one chromosome 1, whereas the homologue showed only one spot on p21. One metaphase showed two spots on p21 on one

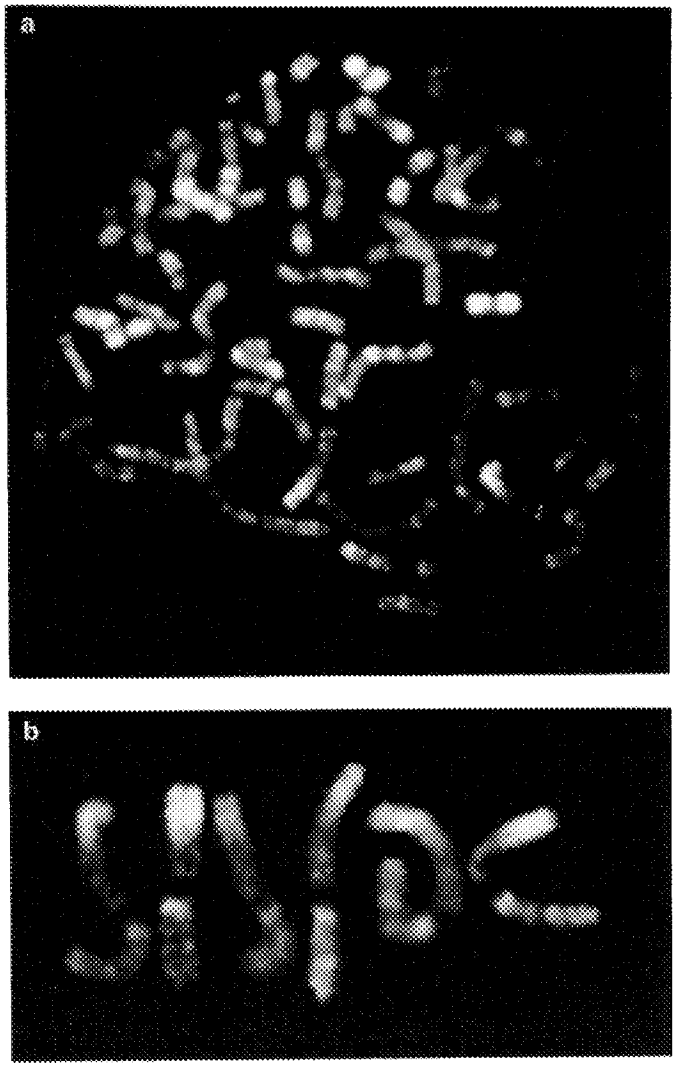


Fig. 5. Chromosomal mapping of HKShIIIC cDNAs. **A:** Double label fluorescence hybridization of a metaphase spread. The R-like banding pattern is generated by hybridization of Alu-PCR products and is pseudocolored in green. The signal from the cDNA clone 2C is pseudocolored in red. **B:** Example of six chromosomes 1 from different metaphase spreads hybridized with cDNA clones 2C or 6A. The red pseudocolored signal from the cDNA clones maps to band p21, identified by Alu-PCR hybridization banding. **C:** Ideogram of chromosome 1. The ideogram presents schematically the R-banding pattern of chromosome 1. The bar beside the ideogram indicates the map position of the cDNA clones.

chromosome 1, the hybridization signal on the homologue, however, was seen at 1p13. Figure 5B shows the signals seen on six different chromosomes 1.

DISCUSSION

Functional Role of HKShIIIC Proteins

Similarly to what has been seen with other Sh cDNAs (Jan and Jan, 1990; Rudy et al., 1991), in vitro transcripts of HKShIIIC express voltage-dependent K^+ channels in *Xenopus* oocytes. These channels are likely to be homomultimers, perhaps tetramers of HKShIIIC proteins. A novel type of A current is seen in *Xenopus* oocytes injected with HKShIIIC transcripts. This current requires large depolarizations for activation and is very sensitive to the channel blocker TEA. The identification of HKShIIIC cDNAs in a brain stem cDNA library suggests that ShIIIC mRNAs are expressed in cells present in this brain region. However, to our knowledge no oocyte-like HKShIIIC current has been described in cells of the brain stem. Such currents may be present in cells or cell regions from which electrophysiological recordings have not been obtained yet, or may have been obscured by other currents in previous experiments. Low-

voltage (−70 to −60 mV) activating A currents regulate firing frequency, the delay between depolarization initiation and burst generation, and other features of neuronal firing patterns (Connor and Stevens, 1971b; Thompson and Aldrich, 1980; Dekin and Getting, 1984; Hille, 1984; Adams and Galvan, 1986; Rudy, 1988; Llinas, 1984, 1988). It has been suggested that transient K^+ currents that start increasing around −40 mV (such as those expressed by some ShI genes) may play similar roles in cells were the depolarizing current (e.g., a Ca^{++} current) is also activated at similar potentials (Rudy, 1988). Interesting functional consequences can be expected if currents like those seen in *Xenopus* oocytes injected with HKShIIIC cRNA are present in native cells. However, as is the case with other Sh proteins, it remains to be shown that homomultimers of these proteins, such as those formed in the *Xenopus* expression system, exist in vivo.

ShI proteins produce novel channels when two ShI cDNAs are coexpressed (Christie et al., 1990; Isacoff et al., 1990; Ruppersberg et al., 1990; McCormack et al., 1990b). These channels are probably heteromultimers of the two ShI proteins. Sh proteins of different subfamilies appear not to form heteromultimers (McCormack et al., 1990b; Covarrubias et al., 1990). We may expect from these studies that HKShIIIC will form heteromultimers with other ShIII proteins. In addition, Sh proteins may undergo posttranslational modifications such as glycosylation and phosphorylation, which could change the properties of Sh channels (Rudy et al., 1991). Channels composed of Sh subunits can apparently also interact with other not yet identified subunits, and this may also change the functional properties of the final product (Rehm et al., 1989a,b; Rehm and Lazdunski 1988; Rudy et al., 1988, 1991). Factors such as those listed here are likely to contribute to the functional properties of native channels containing HKShIIIC proteins. For example, rat KShIIIC RNAs are expressed in PC12 pheochromocytoma cells (Vega et al., 1991a); however, the PC12 cell current that most closely resembles oocyte-like HKShIIIC currents starts activating at lower membrane potentials and inactivates more slowly. The identification of native channels containing HKShIIIC proteins remains a task for the future and a prerequisite to understand their functional role.

The ShIII Gene Subfamily

cDNAs from two Shaw-like (ShIII) genes in mammals had been previously cloned: RKShIIIA (McCormack et al., 1990a) and NGK2 (Yokoyama et al., 1989; RKShIIIB, shown in Fig. 2, is a rat homologue of NGK2). The protein products of these cDNAs are more similar to the products of the *Drosophila* Shaw gene than to other *Drosophila* Sh proteins. However, the percentage of amino acid identity seen between mammalian ShIII proteins and Shaw (49–56%), their *Drosophila* counterpart, is less than that seen between mammalian and fly homologues in the other three subfamilies (70–80%) suggesting faster evolution at some stage. Four ShIII genes in mammals were identified by amplification of human and rat genomic DNA by the polymerase chain reaction (PCR, Sen et al., 1991). HKShIIIC and Raw3 (Schroter et al., 1991) represent the third member of this group for which a complete coding sequence has been obtained. The predicted phylogenetic relationships of these genes, based on sequence alignment and comparison (Rudy et al., 1991), indicate that as in the case of the Shaker-like (ShI) genes, the Shaw-like precursor underwent extensive duplication and subsequent variation in the chordate line. A high amino acid sequence conservation is seen between rodent and human ShI proteins (Ramaswami et al., 1990; Grupe et al., 1990), suggesting strong evolutionary pressures to maintain subtle differences in the structure and function of each protein subtype. We find a similarly high amino acid sequence conservation between HKShIIIC and Raw3 (a rat homologue; Schroter et al., 1991). We have also cloned a human homologue of RKShIIIB, and observe a 99% amino acid sequence identity between the predicted proteins (unpublished observations). It is likely that in both the ShI and the ShIII subfamilies, each protein subtype was incorporated into important and specific functions that cannot be substituted by another subtype. Further evolutionary analysis of these proteins may provide clues to their functional roles.

Structure and Function of Voltage-Gated K^+ Channels

The amino acid sequence and functional similarities and differences between HKShIIIC and other ShIII proteins could contribute to the identification of domains involved in voltage-dependent K^+ channel function. Particularly interesting in this regard is the inactivation of HKShIIIC channels. In inactivating *Drosophila* Shaker channels fast inactivation has been associated with sequences present in the amino domain consisting of clusters of positively charged residues next to hydrophobic amino acids (Hoshi et al., 1990). Internal deletions in the amino domain of Shaker cDNAs result in the expression of noninactivating channels (Hoshi et al., 1990). If the ATG chosen as the start codon is indeed used for translation initiation (Fig. 1), the HKShIIIC protein contains a 28 amino acid insert in the amino end of the protein (Fig. 2). This insert contains several positively charged residues and could thus play a similar role to the domains in the amino end region of Shaker channels. Structure function studies on HKShIIIC could con-

tribute further information towards our understanding of the molecular mechanisms of K^+ channel inactivation.

Comparison of ShIII products with other Sh proteins should also help find sequence domains that may explain why ShIII channels require large depolarizations to activate. A candidate for this is the conserved substitution of one of the leucines in the leucine heptad repeat adjacent to the S4 domain for a phenylalanine (Fig. 2). Substitutions of these leucines have been shown to produce shifts in the voltage-dependence of *Drosophila* Shaker channels (McCormack et al., 1991).

Also interesting is the sensitivity of HKShIIIC channels to TEA. All ShIII proteins contain a tyrosine in the H5 domain (residue 443 in HKShIIIC). A tyrosine in this position has been shown, by site directed mutagenesis of a *Drosophila* Shaker cDNA, to impart high sensitivity to TEA (MacKinnon and Yellen, 1990). However, HKShIIIC channels are about 5 times more sensitive to TEA than RKShIIIA channels (McCormack et al., 1990a). These differences in TEA sensitivity cannot be explained with our present knowledge of the residues involved in TEA binding (MacKinnon and Yellen, 1990). The only difference (see Fig. 2) in the H5 domain between RKShIIIA and HKShIIIC is the residue two positions C-terminal to the tyrosine. RKShIIIA has a glutamine in this position while HKShIIIC has a lysine. However if this residue was close to the TEA binding site one would expect a reduced TEA sensitivity, contrary to our observations. There are several amino acid differences between RKShIIIA and HKShIIIC immediately after the H4 domain. Perhaps these or residues in other parts of the protein account for the distinct TEA sensitivities of the two channels.

ACKNOWLEDGMENTS

We thank J.W. Lin for his tremendous help in many aspects of this work. We thank Celia Campagnoni (UCLA) and Carmie Puckett (CALTECH) for their cDNA libraries. This research was supported by U.S. Public Health Service Grant GM26976 to B.R. and HG-00272 to D.C.W. T.R. is a recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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